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Original Article

Methylthioadenosine phosphorylase represents a predictive marker for response to adjuvant interferon therapy in patients with malignant melanoma

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Abstract: Using tissue microarrays assembling 465 nevi, primary melanomas and metastases, we investigated whether expression of methylthioadenosine phosphorylase (MTAP), a recently suggested biomarker of malignant melanoma, has prognostic significance and may predict responsiveness to adjuvant interferon therapy in patients with melanoma. Because of its association with MTAP activity and interferon signalling pathways, signal transducer and activator of transcription 1 (STAT1) immunohistochemistry was analysed, too. MTAP expression was significantly reduced in melanomas and metastases compared with nevi ($P < 0.001$); STAT1 expression significantly increased. In melanomas, loss of MTAP expression was significantly related to Clark level ($P < 0.05$) and tumor thickness ($P < 0.01$); whereas STAT1 immunoreactivity was significantly related to gender ($p < 0.05$) and tumor thickness ($P < 0.05$). Interestingly, subgroup analysis of patients with a tumor thickness of 1.5–4.0 mm revealed a significant survival benefit from adjuvant interferon treatment regarding recurrence-free survival (RFS; $P < 0.05$) if MTAP expression was observed in the primary melanoma. Patients with STAT1-positive melanomas also tended to benefit from interferon concerning RFS ($P = 0.074$) and showed a significant benefit concerning overall survival (OS; $P < 0.05$). According to Cox analysis, MTAP expression in contrast to STAT1 was an independent positive prognostic marker for RFS and OS. In conclusion, MTAP represents a highly promising immunohistochemical marker for prognosis and interferon response of patients with malignant melanoma.

Key words: interferon alpha – malignant melanoma – methylthioadenosine phosphorylase – signal transducer and activator of transcription 1

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Introduction

Methylthioadenosine phosphorylase (MTAP), which is constitutively expressed in most normal cells and tissues, plays a significant role in polyamine metabolism; the housekeeping enzyme catalyses the phosphorylation of methylthioadenosine (MTA), a by-product of polyamine synthesis (1).

Lack of MTAP activity was shown to occur in several malignant diseases like breast cancer, endometrial cancer, leukaemia, osteosarcoma and hepatocellular carcinoma (2–6). Loss of MTAP activity was related to deletions in human chromosome 9p21, encoding the tumor suppressor genes CDKN2A and CDKN2B, MTAP, and interferon alpha and beta and to epigenetic regulation by promoter hypermethylation (1,7).

In malignant melanomas, selective deletions in this chromosomal region or promoter hypermethylation are known to result in a loss of MTAP protein expression. Immunohistochemistry (IHC) studies revealed a significant inverse association between MTAP protein expression and progression of melanocytic tumors, with the amount of MTAP protein staining decreasing from benign melanocytic nevi to metastatic melanomas (7).

In addition to its role in polyamine metabolism, MTAP expression has significant impact on signal transducer and activator of transcription 1 (STAT1) activity (8). STAT1 is activated by peptide hormones, growth factors and cytokines, particularly cytokines of the interferon family, and is essential for the activation of interferon γ signalling pathways (9). Mowen et al. (2001) postulated an association

between MTAP activity and interferon sensitivity via the transcription factor STAT1 which requires phosphorylation on its serine residue (ser 727) for maximal transcriptional activity induced by interferon γ. Consistently, recent *in vitro* experiments in melanoma cell lines have illustrated that re-expression of MTAP leads to responsiveness to interferon (7).

In our previous work based on a subgroup analysis of a small cohort of patients with melanoma, we suggested that MTAP might be a predictive marker of interferon therapy resistance in patients with melanoma and disease progression (10). As reliable markers for the prediction of therapy outcome of adjuvant interferon treatment are urgently needed, in the tissue microarray study presented here, a large cohort of patients with melanoma was analysed to evaluate whether expression of MTAP and the downstream signal transducer and transcription activator STAT1 are of prognostic or therapeutic relevance in patients with melanoma.

Materials and methods

The medical ethical committee of the University of Regensburg, Germany, approved all described experiments. The retrospective study was conducted according to the Declaration of Helsinki Principles.

Tissue microarrays

Tissue microarrays (TMAs) were constructed as described previously (11) and contained a total of 465 formalin-fixed, paraffin-embedded human tissues from 465 different patients: 364 (78.3%) primary malignant melanomas, 39 (8.4%) metastases and 62 (13.3%) benign nevi. In patients with multiple subsequent neoplasms, only initial and single primary malignant melanomas were included. H&E-stained slides of all tumors were evaluated by two dermatohistopathologists (TV, SM). Clinical follow-up data, provided by the Central Tumour Registry Regensburg, were available for all patients with primary malignant melanomas; for statistical analysis, only patients with a minimum follow-up period of 6 months were considered (n = 360), i.e. four patients with primary malignant melanoma were excluded from further investigation owing to a shorter follow-up period. The median follow up for all patients with malignant melanoma was 51.5 months (range 6–186 months). The University of Regensburg institutional review board granted approval for the project. Characteristic parameters of the TMA are summarised in Table 1 and S1.

Immunohistochemical analysis

Paraffin-embedded preparations of tissues from patients with benign melanocytic nevi, malignant melanomas and melanoma metastases were screened for MTAP and STAT1 protein expression by IHC as described previously (7,10,12). In brief, tissues were deparaffinised, rehydrated and subsequently incubated with primary polyclonal chicken anti-MTAP/monoclonal rabbit anti-STAT1 antibody (1:1.500/1:200) overnight at 4°C. The secondary antibody (biotin-labelled anti-chicken, 1:1.000; Jackson ImmunoResearch Laboratories, Ltd., West Grove, PA/biotin-labelled anti-rabbit, Zytomed Systems, Berlin, Germany) was incubated for 30 min at room temperature, followed by incubation with streptavidin-peroxidase (Dako Cytomation GmbH, Hamburg, Germany) for 30 min. The following primary antibodies were used: anti-MTAP (generous gift from Dr D. Carson, University of California), anti-STAT1 (rabbit monoclonal; Cell Signalling; 1:200; final concentration 10 μg/ml) and anti-Ki67 (rabbit monoclonal, clone MIB1; Dako; 1:10, final concentration 5 μg/ml). As an internal positive control for MTAP IHC, normal squamous epithelium of the epidermis was chosen; human colon carcinoma tissue served as positive

Table 1. MTAP expression analysis of melanocytic skin tumors

Melanocytic lesions (n = 461)	Cytoplasmic MTAP immunoreactivity[1]				Cytoplasmic STAT1 immunoreactivity[2]			
	n analysable[3]	score 0 (n)	Score 1+ to 2+ (n)	P*	n analysable[4]	Score 0 (n)	Score 1+ to 3+ (n)	P*
Total	392	75	317	**<0.001**	440	276	164	**<0.001**
Primary malignant melanomas[5]	297	55	242		341	194	147	
Melanoma metastases	34	19	15		39	24	15	
Benign nevi	61	1	60		60	58	2	

MTAP, methylthioadenosine phosphorylase; STAT1, signal transducer and activator of transcription 1.
*Chi-square test; boldface indicates statistical significance.
[1]MTAP staining intensity (0 to 2+): 0, negative; 1+, weak positive; 2+, strong positive.
[2]STAT1 staining intensity (0 to 3+): 0, negative; 1+, weak positive; 2+, strong positive; 3+, very strong positive.
[3]Missing data because of missing values: n = 69.
[4]Missing data because of missing values: n = 21.
[5]Only initial and single primary malignant melanomas were included.

control for STAT1 IHC. Antibody binding was visualised using AEC solution (Dako). Tissues were counterstained with hemalaun. A surgical pathologist (FB) and a dermatohistopathologist (SM) performed a blinded evaluation of the stained slides. According to Wild et al. (2006), cytoplasmic MTAP staining intensity was estimated using a semiquantitative three-step scoring system (0 to 2+): 0, negative; 1+, weak positive; 2+, strong positive. Cytoplasmic STAT1 staining intensity was estimated using a semiquantitative four-step scoring system (0 to 3+): 0, negative; 1+, weak positive; 2+, strong positive, 3+, very strong positive. In consideration of the small amount of tissue of each individual tumor on the TMA, even weak MTAP and STAT1 immunoreactivity (1+) was considered positive. The percentage of Ki-67-positive cells of each specimen was determined as described previously (13). High-Ki-67 labelling index was defined if at least 5% of the tumor cells were positive.

Statistical analysis

All specimens on the TMAs were considered independently. Regarding the patients with primary melanomas, only cases with a minimum follow-up period of 6 months were included. Contingency table analysis, two-sided Chi-square and Fisher's exact tests were used to study the statistical association between clinicopathological and immunohistochemical parameters. Retrospective overall survival (OS) and recurrence-free survival (RFS) curves comparing patients with or without any of the factors were calculated using the Kaplan-Meier method, with significance evaluated by two-sided log rank statistics. For the analysis of RFS, patients were censored at the time of their last tumor-free clinical follow-up appointment. For OS analysis, patients were censored at the time of their last clinical follow-up appointment or at their date of death not related to the tumor. P values <0.05 were considered significant. Statistical analyses were completed using SPSS version 15.0 (SPSS, Chicago, IL, USA). Multivariable Cox regression models for OS and RFS were adjusted, testing the independent prognostic relevance of MTAP and STAT1 immunoreactivity, with tumor thickness as a continuous and status of interferon therapy as dichotomous variable. Further models including the interaction between MTAP and interferon therapy (respectively STAT1 and interferon therapy) were analysed. As these analyses were exploratory in nature and further validation of our results is hence necessary, no adjustments for multiple testing were made in order to avoid loss of statistical power.

Results

Immunohistochemical analysis

Using TMA technology, investigation of MTAP and STAT1 protein expression was informative in 392 (85%) of 461 and 440 (95%) of 461 cases, respectively (data were missing for 69 and 21 cases). MTAP and STAT1 protein expression of any intensity was detected in 317 (81%) of 392 and in 164 (37%) of 440 informative cases. Table 1 summarises the IHC results for each tumor entity on the TMA. Compared with benign nevi, expression of MTAP was significantly reduced in malignant melanomas and melanoma metastases ($P < 0.001$); in contrast, expression of STAT1 significantly increased ($P < 0.001$). MTAP and STAT1 immunoreactivity were not significantly associated ($P = 0.268$).

Clinicopathological variables were compared relative to MTAP and STAT1 expression (Table S1). In primary melanomas, loss of MTAP expression was significantly related to higher Clark levels ($P < 0.05$) and greater tumor thickness ($P < 0.01$). Increased STAT1 expression was significantly associated with female gender ($P < 0.05$) and a tumor thickness of 1.5–4.0 mm ($P < 0.05$). On the TMA, no differences in MTAP and STAT1 expression were observed between compound and dermal nevi.

Prognostic relevance

Using univariate log rank statistics, OS and RFS were compared between MTAP-positive and -negative cases, and STAT1-positive and -negative cases (Table 2). In patients with primary malignant melanomas, expression of MTAP was significantly associated with OS ($P < 0.01$) (Fig. 1a) and RFS ($P < 0.05$) (Fig. 1b), respectively. For the expression of STAT1, no significant prognostic relevance was found regarding OS ($P = 0.160$) and RFS ($P = 0.492$).

According to a global Cox regression model without variable selection, expression of MTAP represented an independent positive prognostic marker for both RFS (hazard ratio 0.361, 95% confidence interval 0.172–0.758, $P < 0.01$; Table S2a) and OS (hazard ratio 0.554, 95% confidence interval 0.319–0.962, $P < 0.05$; Table S2b); in contrast to expression of STAT1. Interferon therapy and tumor thickness showed significant influences in both Cox models.

Predictive relevance

To evaluate the therapeutic benefit, a subgroup analysis was performed among the melanoma patients with a tumor thickness of 1.5–4 mm with (low-dose interferon alpha: three million units, administered subcutaneously three times a week, mostly for 24 months) and without adjuvant interferon treatment. Interferon alpha therapy is recommended in an adjuvant setting for patients with melanoma with high risk of recurrence, i.e., a minimum tumor thickness of 1.5 mm without clinically detectable lymph node metastases and after resection of lymph node metastases corresponding to stages II to III (AJCC 2002), respectively (http://www.ado-homepage.de/projekte/1/upload/kurzleitli-

Table 2. Univariate analysis of clinicopathological variables relative to recurrence-free survival (RFS) and overall survival (OS) among patients with primary malignant melanoma (n = 360)

Variable	Categorisation	Tumor recurrence (RFS)			Tumor-related death (OS)		
		n^1	Events	P*	n^1	Events	P*
Age at diagnosis	≤60 years	176	33	0.202	178	42	0.124
	>60 years	180	20		182	53	
Gender	Female	164	20	0.914	168	32	**0.003**
	Male	192	33		192	63	
Clark level[2]	I	1	0	**<0.001**	1	0	**<0.001**
	II	74	2		75	8	
	III	106	6		106	11	
	IV	143	34		146	59	
	V	14	5		14	11	
Tumor thickness	<1.5 mm	206	9	**<0.001**	209	24	**<0.001**
	1.5–4.0 mm	96	22		96	35	
	>4.0 mm	48	20		49	33	
Nodal status	pN0	314	42	**<0.01**	317	74	**<0.001**
	pN1	11	6		11	8	
	pN2	6	2		6	5	
	pN3	4	2		4	3	
Ki67 labelling index	≤5%	199	23	**<0.05**	200	48	0.205
	>5%	139	27		142	41	
Cytoplasmic MTAP IHC	Score 0	53	11	**<0.05**	55	19	**<0.01**
	Score 1+ to 2+	240	36		242	63	
Cytoplasmic STAT1 IHC	Score 0	192	31	0.492	194	57	0.160
	Score 1+ to 3+	145	19		147	32	

MTAP, methylthioadenosine phosphorylase; IHC, Immunohistochemistry; STAT1, signal transducer and activator of transcription 1.
*Log rank test (2-sided), boldface indicates statistical significance.
[1]only initial and single primary malignant melanomas were included (numbers <360 because of missing values).
[2]taken from Sobin LH, Wittekind CH, eds. UICC: TNM Classification of Malignant Tumours. 6th edn. New York, NY: John Wiley & Sons Inc; 2002.

niemelanom20063.pdf). The upper threshold of tumor thickness was defined at 4.0 mm as tumors with a thickness ≥4 mm are rarely observed (14), and the almost linear correlation between tumor thickness and melanoma-specific mortality rate is lost beyond 4 mm (15). This subgroup of patients was considered to be most representative for the majority of patients with melanoma receiving adjuvant treatment, and therefore chosen for further analysis.

In this subgroup of R0 resected patients with a tumor thickness of 1.5–4 mm (UICC stage IB: five patients, stage II: 24 patients, stage III: three patients, stage IV: three patients, missing stage: four patients), patients with MTAP-positive primary melanomas treated with interferon had a significantly longer RFS ($P < 0.05$) compared to patients with MTAP-negative tumors (Fig. 2a) and tended to show a longer OS (median survival 80 months versus 35 months

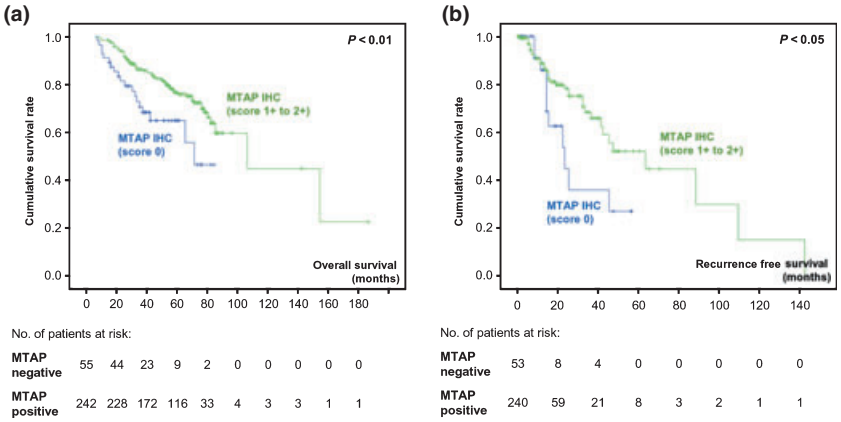

Figure 1. Methylthioadenosine phosphorylase (MTAP) expression is associated with prognosis of patients with melanoma. In patients with primary malignant melanomas, expression of MTAP was significantly associated with (a) overall survival (n = 297; $P < 0.01$) and (b) recurrence-free survival (n = 293; $P < 0.05$) as estimated by the Kaplan-Meier method.

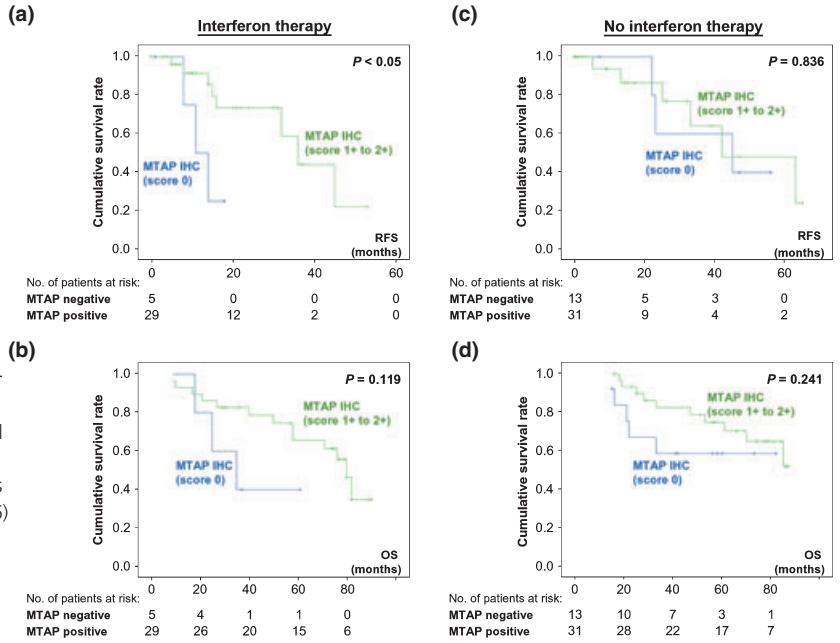

Figure 2. Methylthioadenosine phosphorylase (MTAP) expression is correlated with interferon therapy response of patients with melanoma. In patients with primary malignant melanomas (tumor thickness: 1.5–4 mm), expression of MTAP was significantly associated with recurrence-free survival in patients (a) receiving adjuvant interferon treatment ($n = 34$; $P < 0.05$) in contrast to patients (c) without interferon treatment ($n = 44$; $P = 0.836$) as estimated by the Kaplan-Meier method; MTAP expression was not significantly associated with overall survival in patients (b) with ($n = 34$; $P = 0.119$) and (d) without interferon therapy ($n = 44$; $P = 0.241$).

for MTAP-negative melanomas; $P = 0.119$) (Fig. 2b). There was no significant prognostic relevance comparing patients with MTAP-positive and -negative tumors who did not receive interferon treatment concerning RFS ($P = 0.836$) (Fig. 2c) or OS ($P = 0.241$) (Fig. 2d). Patients with STAT1-positive melanomas with interferon treatment did not show a significant benefit concerning RFS ($P = 0.074$) (Fig. 3a), but showed a calculated benefit concerning OS ($P < 0.05$) (Fig. 3b). There was no prognostic relevance comparing patients with STAT1-positive and -negative tumors who did not receive interferon treatment regarding RFS ($P = 0.203$) (Fig. 3c) and OS ($P = 0.512$) (Fig. 3d).

Patients with melanoma, with a tumor thickness >4 mm with and without interferon therapy did not show a significant survival benefit concerning OS or RFS regardless of MTAP or STAT1 immunoreactivity (data not shown).

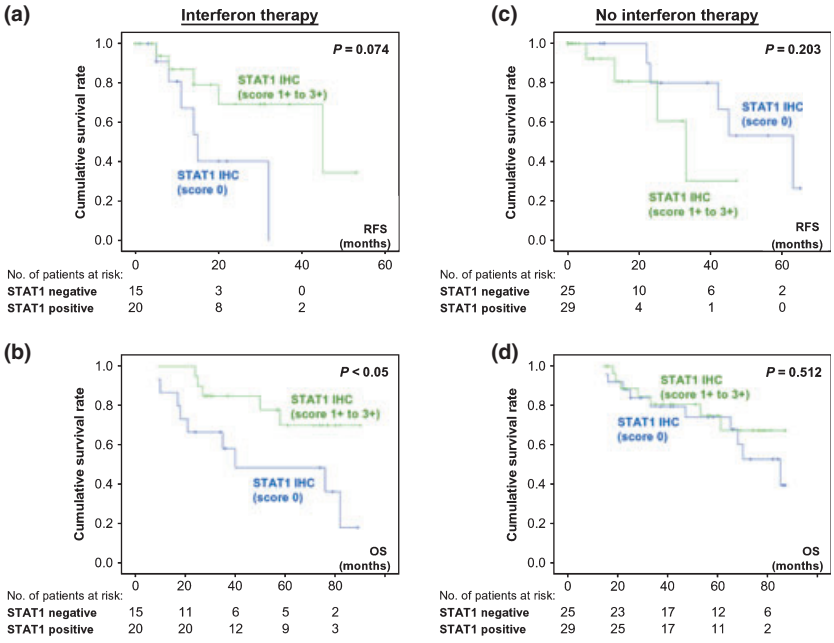

Figure 3. Signal transducer and activator of transcription 1 (STAT1) expression and interferon therapy response of patients with melanoma. In patients with primary malignant melanomas (tumor thickness: 1.5–4 mm), expression of STAT1 was not associated with (a) recurrence-free survival ($n = 35$; $P = 0.074$) but significantly associated with (b) overall survival ($n = 35$; $P < 0.05$) in patients who received adjuvant interferon treatment as estimated by the Kaplan-Meier method; expression of STAT1 was not significantly associated with (c) recurrence-free survival ($n = 54$; $P = 0.203$) or (d) overall survival ($n = 54$; $P = 0.512$) in patients who did not receive interferon treatment.

Discussion

This study clearly shows that MTAP protein expression of primary malignant melanomas is of prognostic relevance and predictive for adjuvant interferon therapy response in patients with primary melanomas.

A significant inverse association between MTAP protein expression and progression of melanocytic tumors was recently shown by immunohistochemical TMA analysis of tissue samples of 80 malignant melanomas, 95 melanoma metastases and 137 benign nevi that revealed decreasing amounts of MTAP protein staining in the progression from benign nevi to metastatic melanomas (10). In this former, smaller study, MTAP immunoreactivity was not associated with prognosis regarding OS or RFS. Based on a subgroup analysis within this study performed among 13 patients with MTAP-negative and 26 patients with MTAP-positive melanomas with tumor recurrence, patients with MTAP-negative melanomas with tumor recurrence and interferon treatment showed a significantly reduced OS compared to patients with MTAP-positive melanomas with tumor recurrence and interferon treatment ($P < 0.01$). In conclusion, loss of MTAP protein expression was suggested to be a predictive marker for interferon therapy resistance in patients with melanoma and disease progression (10). This implicates that MTAP inactivation might affect tumor response to interferon therapy in patients with melanoma. To validate these data, further quantification of MTAP expression in a larger cohort of patients with melanoma was of particular interest as reliable markers for prediction of therapy outcome are urgently needed.

According to Mowen et al. (2001), MTAP activity and interferon sensitivity are associated via STAT1, a signal transducer and transcription factor downstream of MTAP, which modulates interferon α/β-induced transcription. Reduction of MTAP activity in the cell leads to accumulation of MTA, which acts as an inhibitor of methyltransferases. Methylation of arginine 31 in STAT1 by protein arginine *N*-methyltransferase 1 (PRMT1) was found to be an important modification. Loss of this modification leads to enhanced binding of protein inhibitor of activated STAT (PIAS1) to STAT1, and, therefore to inhibition of STAT1 DNA binding activity. As STAT1 is essential for interferon signalling pathways, loss of MTAP was expected to reduce the cell response to interferon treatment. Based on this postulate, we analysed the expression of STAT1 for its prognostic and predictive relevance in addition to MTAP expression.

In contrast to our previous study including 80 patients with primary malignant melanomas (10), in this large TMA study based on a total of 360 patients with melanoma with a minimum follow-up period of 6 months, MTAP immunoreactivity was associated with prognosis (i.e. OS and RFS). For expression of STAT1, however, no significant prognostic relevance was found regarding OS ($P = 0.160$) and RFS ($P = 0.492$) (see Table 2). Moreover, no significant influence of STAT1 was found on RFS or OS in global Cox regression models in contrast to MTAP. Summing up, compared with the transcription factor STAT1, which is located downstream of MTAP in interferon signalling pathways in different modes of activation, MTAP represents a promising candidate to predict individual prognoses of patients with melanoma. Recently, Wang et al. (16) suggested that the relative balance of pSTAT1/pSTAT3 may be associated with melanocyte differentiation *in vivo*, and pSTAT3 is a potential biomarker of melanocytic transformation and progression which is modulated by interferon alpha dose-dependently. Accordingly, STAT3 was suggested to be a potential target for chemoprevention of melanoma. Considering these aspects, it would be of particular interest to study other candidates in the Janus-activated kinase/signal transducers and activators of transcription (STAT) pathway of interferon signalling in relation to MTAP expression and the therapeutic impact of interferon.

Owing to the evident need of predictive markers for therapy outcome, we subdivided the collective into groups with and without adjuvant interferon treatment. In addition, patients with primary malignant melanomas with a tumor thickness of 1.5–4.0 mm (pT2-3) and larger than 4.0 mm (pT4) were analysed in parallel to evaluate the predictive impact of MTAP and STAT1 immunoreactivity regarding responsiveness to adjuvant interferon treatment. Consistent with the findings of Mowen et al. (2001) and our previous observations (10), in the small but representative subgroup of patients analysed in this retrospective study patients with MTAP-positive melanomas with a tumor thickness of 1.5–4.0 mm had a significant benefit from interferon treatment regarding RFS ($P < 0.05$) and tended to benefit from interferon treatment regarding OS (median survival 80 months versus 35 months for MTAP-negative melanomas; $P = 0.119$), whereas patients with STAT1-positive melanomas (tumor thickness of 1.5–4.0 mm) tended to benefit from interferon treatment concerning RFS ($P = 0.074$) and showed a significant benefit concerning OS ($P < 0.05$). Interestingly, no predictive value was found for patients with tumors larger than 4 mm pointing to enhanced aggressiveness probably because of additional, yet poorly understood molecular changes and pathways involved in the biology of very thick primary malignant melanomas.

Compared to STAT1, a transcription factor with different modes of activation located downstream of MTAP in interferon signalling pathways, determination of the MTAP immunoreactivity in primary malignant melanomas prior to interferon treatment seems to be an appropriate tool to pre-estimate a patient's chance to benefit from interferon therapy.

Our data, supporting the previously suggested association between MTAP activity and interferon sensitivity, may be of particular clinical importance as biological markers predictive for tumor response to adjuvant interferon therapy have not yet been defined. Up to now, interferon alpha is the only therapeutic agent in adjuvant treatment of malignant melanomas showing a significant (metastasis free) survival benefit in prospective randomised clinical trials (17,18). As previously mentioned, adjuvant therapy with interferon alpha should be recommended to all patients with melanoma with high risk of recurrence (tumor thickness >1.5 mm and/or lymph node metastases) unless there are clear contraindications. Any adjuvant treatment with interferon may have serious side effects and reduces the quality of life. Hence, the indications for interferon treatment have to be carefully assessed. For systematic selection of high-risk patients with melanoma with a predictable chance to benefit from interferon therapy, immunohistochemical profiling of primary malignant melanomas responding to interferon treatment would be of major clinical interest.

According to the data presented here, MTAP represents a highly promising immunohistochemical marker to predict a patient's prognosis and responsiveness to adjuvant interferon treatment. Prospective clinical trials will validate the predictive value of MTAP immunoreactivity for routine clinical assessment of primary malignant melanomas regarding response to interferon treatment. There is a compelling rationale for new research upon targeted antitumor therapies (19) including interferon response, especially in adjuvant settings as recently summarised (18). Scientific approaches that may enable practitioners to determine which patients may benefit from interferon therapy are essential for a patient-oriented therapy of malignant melanoma and indispensable from the health economic point of view.

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Conflict of interest

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1. Clinicopathological variables and MTAP-/STAT1-expression among analysable tissues (n = 461).

Table S2a. COX regression analysis for recurrence-free survival of patients with primary malignant melanomas.

Table S2b. COX regression analysis for overall survival of patients with primary malignant melanomas.

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